# Genetic characterization of *Strongyloides* spp. from captive, semi-captive and wild Bornean orangutans (*Pongo pygmaeus*) in Central and East Kalimantan, Borneo, Indonesia

E. M. LABES[1], N. WIJAYANTI[2], P. DEPLAZES[1] *and* A. MATHIS[1]*

[1] *University of Zurich, Institute of Parasitology, Winterthurerstrasse 266a, 8057 Zurich, Switzerland*
[2] *University Gadjah Mada, Centre for Biotechnology, 55281 Jogjakarta, Indonesia*

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SUMMARY

Orangutans (*Pongo* spp.), Asia's only great apes, are threatened in their survival due to habitat loss, hunting and infections. Nematodes of the genus *Strongyloides* may represent a severe cause of death in wild and captive individuals. In order to better understand which *Strongyloides* species/subspecies infect orangutans under different conditions, larvae were isolated from fecal material collected in Indonesia from 9 captive, 2 semi-captive and 9 wild individuals, 18 captive groups of Bornean orangutans and from 1 human working with wild orangutans. Genotyping was done at the genomic rDNA locus (part of the 18S rRNA gene and internal transcribed spacer 1, ITS1) by sequencing amplicons. Thirty isolates, including the one from the human, could be identified as *S. fuelleborni fuelleborni* with 18S rRNA gene identities of 98·5–100%, with a corresponding published sequence. The ITS1 sequences could be determined for 17 of these isolates revealing a huge variability and 2 main clusters without obvious pattern with regard to attributes of the hosts. The ITS1 amplicons of 2 isolates were cloned and sequenced, revealing considerable variability indicative of mixed infections. One isolate from a captive individual was identified as *S. stercoralis* (18S rRNA) and showed 99% identity (ITS1) with *S. stercoralis* sequences from geographically distinct locations and host species. The findings are significant with regard to the zoonotic nature of these parasites and might contribute to the conservation of remaining orangutan populations.

Key words: *Strongyloides fuelleborni*, *Strongyloides stercoralis*, genomic rDNA locus, Bornean orangutan, *Pongo pygmaeus*, zoonosis.

## INTRODUCTION

Orangutans (family *Pongidae*) represent the only great ape in Asia. The 2 existing species inhabit the islands Borneo (*Pongo pygmaeus*) and Sumatra (*Pongo abelii*) in Indonesia and Malaysia (Rijksen and Meijaard, 1999). Due to human activities such as logging and hunting, total population numbers are rapidly decreasing. In 1997, the population of Bornean orangutans was estimated to be down to 7% of the population in 1900 (Rijksen and Meijaard, 1999), and the species is classified as endangered by the IUCN (2010). Re-introduction programmes in Borneo aim at releasing confiscated orangutans into protected forest habitats. These programmes rely on re-introduction centres where large numbers of individuals are kept over several years. The health of orangutans is affected by a wide variety of intestinal parasites among which nematodes of the genus *Strongyloides* are of major importance. Infections can be fatal and may represent a major cause of death in captive individuals (Warren, 2001). Especially young orangutans are considered to be susceptible to developing strongyloidosis (Wells *et al*. 1990), and prevalences in these individuals are high (e.g. 54% in rehabilitating orangutans $\leqslant$5years of age; Labes *et al*. 2010). *Strongyloides* has also been reported from wild orangutan populations (Collet *et al*. 1986; Djojoasmoro and Purnomo, 1998; Warren, 2001) but only few data exist on the occurrence and distribution of *Strongyloides* species or subspecies in these apes. Of the more than 50 species of *Strongyloides* that have been described so far (Grove, 1989; Dorris *et al*. 2002) and which parasitize a wide range of vertebrate hosts, 2 species are of particular importance to humans and non-human primates. *Strongyloides stercoralis* is primarily a human parasite in the southern hemisphere (Fisher *et al*. 1993; Gilles and Cook, 1996) but infections have occasionally also been described in non-human primates (Grove and Northern, 1982; Genta, 1989) causing severe disease or death. Examples include a young orangutan in an English zoo, fatal infections in 2 Bornean orangutans, and 1 rhesus macaque at a US Primate Research Centre (see Grove, 1989). *Strongyloides fuelleborni* occurs naturally in non-human primates but is widespread also in human populations in Africa and Southeast Asia (Nolan *et al*. 1998; Viney, 2006; Viney and Lok,

* Corresponding author: University of Zurich, Institute of Parasitology, Winterthurerstrasse 266a, 8057 Zurich, Switzerland. Tel: +41 (0)44 635 85 01. Fax: +41 (0)44 635 89 07. E-mail: alexander.mathis@uzh.ch

 
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Table 1. Features of the orangutan cohorts investigated

a. Individual animal at centres and field sites

| Captive status | Number of individuals | Length of stay at centre (⩽21 days[1]/>21 days) | |
|---|---|---|---|
| Captive[2] | 9 | 2/7 | |
| Semi-captive[3] | 2 | 0/2 | |
| Wild[4] | 9 | – | |
| Total | 20 | | |
| Age classes | Centre 1 | Centre 2 | Wild |
| Adults (>8 yrs) | 2 | 1 | 7 |
| Subadults (6–8 yrs) | 1 | 1 | – |
| Infants (⩽5 yrs) | 5 | 1 | 1 |
| Unknown | – | | 1 |
| Sex of adults | | | |
| Females | – | 1 | 1 |
| Males | 3 | 1 | 6 |

b. Animal groups at centres

| | Centre 1[5] | Centre 2[6] | Length of stay at centre (⩽21 days[1]/>21 days) |
|---|---|---|---|
| Captive | 6 | 8 | 0/14 |
| Semi-captive | 4 | – | 0/4 |
| Total | 10 | 8 | |

[1] "new arrival"; [2] confiscated, mostly young individuals; [3] wild, mostly adult individuals rescued from oil-palm plantations; [4] free-ranging populations at 3 field sites in the province of Central Kalimantan; [5] orangutan re-introduction centre in Central-Kalimantan; [6] orangutan re-introduction centre in East-Kalimantan.

2007). Two subspecies have so far been described morphologically. *Strongyloides f. fuelleborni* mainly occurs in African primates and humans. *Strongyloides f. kellyi* was identified in Papua New Guinea with a high prevalence, especially in infants without an obvious non-human primate source (Ashford *et al.* 1992). However, molecular phylogenetic analysis did not support the status of a subspecies (Dorris *et al.* 2002).

Several cross-species transmission experiments have confirmed the zoonotic character of *S. f. fuelleborni* which included several monkey species, humans and also dogs (reviewed by Grove, 1989). Both species have also been identified under natural conditions in humans in Zambia with prevalences of 67% for *S. stercoralis* and 30·8% for *S. fuelleborni* (Hira and Patel, 1980).

Identification of *Strongyloides* is based on morphological criteria of the adult female and the fact that either first-stage larvae (*S. stercoralis*) or eggs (*S. fuelleborni*) are excreted with the feces (Little, 1966). Although specific morphological and morphometric characters, particularly in the parasitic female, seem to be robust, strain variation, within-population variability, variation according to age, environmental conditions, host or geographical origin may have an effect on the unequivocal identification of species (effect of intra- and interspecific variability and/or of external factors, respectively) (Grove, 1989). Through molecular analysis, additional (sub-)species of *Strongyloides* might be identified in orangutans since these methods offer much more in-depth investigation.

The aim of this study was therefore to genetically characterize *Strongyloides* infecting wild and captive/semi-captive Bornean orangutans in order to get insight into the occurrence and distribution of these nematodes in the ape which might offer valuable clues as how to protect these endangered animals, as well as humans, from infections.

## MATERIALS AND METHODS

### *Examined individuals*

Fecal samples from 20 individual Bornean orangutans and from 18 groups (2–28 individuals per group, total $n=97$) were investigated (Table 1). Between 1 and 3 samples per individual or group were examined. In addition, a *Strongyloides* isolate from a person working at one of the field-sites was investigated.

### *Study sites*

One of the 2 re-introduction centres (centre 1) is located 28 km north of Palangka Raya (2°21′56·27″S, 113°55′12·48″E), the capital of the province Central

Kalimantan. The centre neighbours a 62·5 ha lowland swamp forest. The second re-introduction centre (centre 2) is located in the centre of a village, 35 km north of the city Balikpapan (1°16′01·03″S, 116°50′01·46″E) in the province East Kalimantan, Borneo. Samples from free-ranging (wild) orangutans were collected from 3 distinct populations in the province of Central Kalimantan (2°09′06·1″S, 114° 26′26·3″E; 2°19′40·72″S, 113°54′39·74″E). All 3 field sites consisted of disturbed forest habitat which had previously been subject to selective logging. Because of their different geographical location, the majority of orangutans at centre 1 belonged to the subspecies *P. pygmaeus wurmbii*, whereas centre 2 had more individuals belonging to the subspecies *P. pygmaeus morio*. The 3 field sites were inhabited by *P. pygmaeus wurmbii*.

### *Collection of larvae*

*Strongyloides* larvae were isolated from fecal material with the Baermann technique. Larvae were identified microscopically to genus level based on the morphological characteristics of either the first- or third-stage larvae (L1: 316·6 ± 104·1 × 18·3 ± 10·4 $\mu$m, rhabditiform oesophagus, short buccal capsule, genital primordium in posterior half of total length; L3: 566·7 ± 57·7 × 26·7 ± 5·8 $\mu$m, filariform oesophagus, slit tail). Isolated larvae were conserved in 75–95% (v/v) ethanol in 1·5 ml centrifuge tubes.

### *DNA extraction from isolated larvae*

After centrifugation of the larvae for 5 min at 5000 ***g***, the supernatant was removed and 200 $\mu$l of 50% (w/v) Chelex (Bio-Rad Laboratories, Reinach, Switzerland) were added per tube. The tubes were vortexed and larval cells were lysed by 4 cycles of freezing/thawing (liquid nitrogen for several sec and boiling in 100 °C heater for 1 min). The tubes were centrifuged at 13 000 ***g*** for 2 min, and the supernatant containing the DNA was transferred into new 1·5 ml centrifuge tubes. Genomic DNA was isolated using a commercial kit according to the manufacturer's instructions (Qiamp DNA mini kit, Qiagen, Hilden, Germany) and were stored at −20 °C until PCR was performed.

### *Amplification, sequencing and sequence analysis*

Parts of the 18S rRNA gene were amplified by PCR using primers StrongF (5′-ATTGATAGCTCTTTCATGATTTAG-3′) and StrongR (5′-AACAGGAACATAATGATCACTAC-3′) which were designed to be specific for the genus *Strongyloides* and which span a variable region. Amplification of the rDNA internal transcribed spacer 1 (ITS1) was done with primers ITS1-F and 5.8S-R as described (Sato *et al*. 2006). PCRs were done in 100 $\mu$l assays containing 5 $\mu$l of template DNA sample, buffer (50 mM KCl, 20 mM Tris-HCl, pH 8·4, 2·5 mM $MgCl_2$, 0·5% (v/v) Tween 20), each deoxynucleoside triphosphate at a concentration of 0·2 mM (with dUTP replacing dTTP) (all reagents from Sigma-Aldrich, Buchs, Switzerland) and each primer at a concentration of 1 $\mu$M. In order to prevent PCR carry-over contamination, 0·5 U of uracil DNA glycosylase (Longo *et al*. 1990) was also included in the reactions, aerosol-guarded tips were consistently used, and DNA isolation, PCR and amplicon analyses were performed in strictly separated laboratories. An initial step at 37 °C for 10 min was performed in an automatic thermal cycler (DNA engine, MJ Research, Waltham, MA, USA) and after 10 min of heat inactivation of the uracil DNA glycosylase, 2·5 U of *Taq* polymerase were added in a hot start. Each PCR cycle of 40 in total consisted of denaturation for 30 sec at 94 °C, annealing for 30 sec at 58 °C, and elongation for 30 sec at 72 °C. Amplicons were detected in 1·5% (w/v) agarose gel, following staining with ethidium bromide. Sequencing was carried out by a private company (Synergene Biotech, Schlieren, Switzerland) either directly or on the cloned (Topo TA cloning-vector pCR 2.1, Invitrogen, Carlsbad, CA, USA) amplicons. DNA isolation, PCRs and sequencing were done once per specimen.

Sequences were aligned using ClustalW, and neighbour-joining trees were constructed using MEGA version 4 (Tamura *et al*. 2007) with the gap opening penalty set to 10 and the gap extension penalty to 3.

## RESULTS

From a total of 30 *Strongyloides* isolates, part of the 18S rRNA gene was determined by PCR/direct sequencing (sequence reads between 134 and 400 bp). With 1 exception from centre 1, all sequences originating from 9 captive, 2 semi-captive and 9 wild orangutans as well as the one derived from a human clustered with a published sequence of *S. fuelleborni fuelleborni* (GenBank AB272235), originating from a Japanese macaque (*Macaca fuscata fuscata*), with sequence identities between 98·5% and 100%. The sequences differed by 5–7% from the corresponding published sequences of *S. procyonis, S. robustus, S. callosciureus, S. cebus, S. stercoralis* and *S. ratti*. A single isolate (no. 9), originating from a captive orangutan, which had arrived at the centre 1 week prior to sample collection, had a sequence identical to an available one of *S. stercoralis* (AF279916).

PCR spanning the rDNA ITS1 region was successful, with 19 *Strongyloides* isolates derived from orangutans (male and female captive, semi-captive animals, kept at both centres either

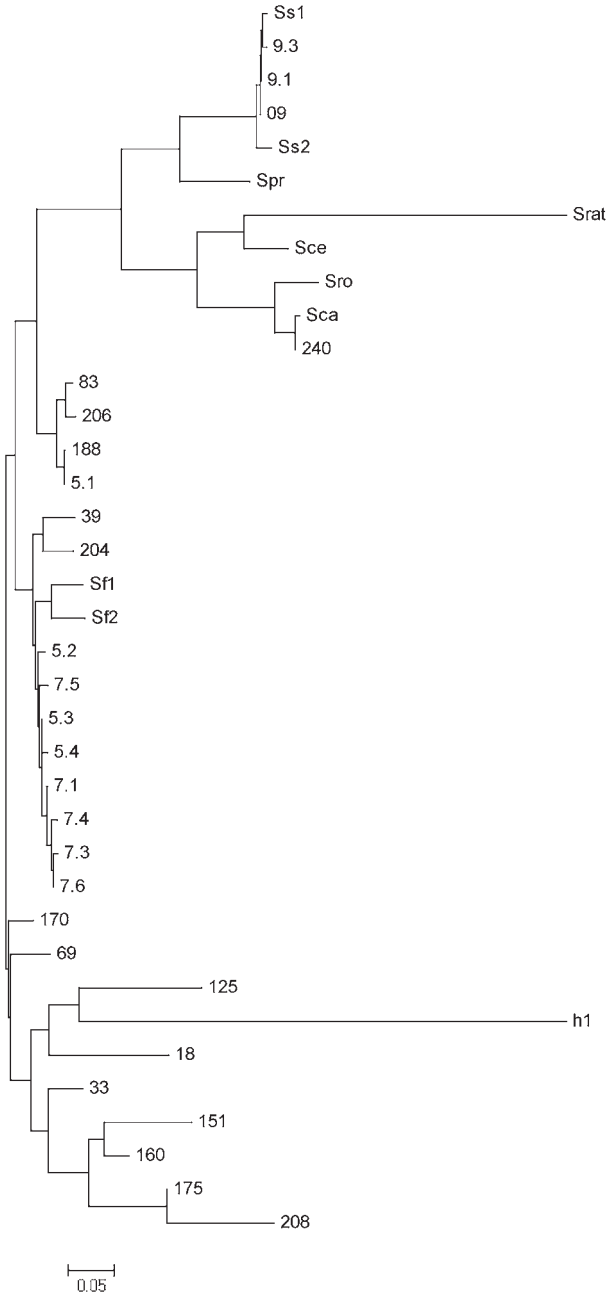


Fig. 1. Dendrogram of rDNA ITS1 sequences of *Strongyloides* spp. Numbers: directly sequenced amplicons of isolates from Bornean orangutans, decimal numbers: sequences of cloned amplicons; h1: directly sequenced amplicon from an isolate from a human. Isolates 5 and 7 could not directly be sequenced; the 4 sequences obtained from the cloned amplicon of isolate no. 5 (5·1–5·4) all had unique sequences; the 6 sequences of isolate no. 7 revealed 5 different sequences. The amplicon of isolate 9 could directly be sequenced, and 4 of 5 sequences of the cloned amplicon were identical to this directly obtained sequence (the sixth one differing at a single polymorphic site). Published sequences (GenBank) include *S. fuelleborni fuelleborni* (Sf1) (AB272235, from captive *Macata f. fuscata* from Japan), *S. fuelleborni fuelleborni* (Sf2) (U43581, undisclosed origin); *S. stercoralis* (Ss1) (U43576, U43578; U43579; identical sequences from isolates from East Asia, Caribbean, USA); *S. stercoralis* (Ss2) (U43962, USA); *S. callosciureus* (Sca) (AB272230), *S. cebus* (Sce) (AB272236), *S. robustus* (Sro) (AB272232), *S. procyonis* (Spr) (AB205054). Nucleotide sequence data reported in this paper are available in the GenBank™ under accession numbers JF699139 - JF699167.

individually or in groups, and wild) and from 1 human. Direct sequence reads between 157 and 387 bp were obtained from 17 isolates; the sequences of isolate nos. 5 and 7 could only be determined after cloning. ITS1 sequences of the 18 isolates that were assigned to *S. fuelleborni fuelleborni* based on partial 18S rRNA gene sequences, including the isolate from the human, were highly variable, being 81–98% identical with the corresponding sequences of this species deposited in GenBank (U43581, AB272235) (Fig. 1). Furthermore, the 4 sequences determined from cloned amplicons of isolate no. 5 (designated 5·1–5·4 in Fig. 1) displayed variability at 17 polymorphic sites, and 4 of the 5 sequenced clones of isolate no. 7 revealed unique sequences. Direct sequencing of the amplicon of isolate no. 9 (*S. stercoralis*) revealed 99% identity (2 polymorphic sites from total 203) as compared with GenBank entries U43576, U43578 and U43579. This amplicon was also cloned, and 4 of the 5 sequences were identical to the direct sequence, whereas the 5th sequence (9·3, Fig. 1) differed at a single polymorphic site.

## DISCUSSION

In this study, primers specifically designed to cover variable regions of the 18S rRNA gene of the genus *Strongyloides* and primers amplifying the ITS1 locus combined with sequencing (either directly or after cloning) were used to identify *Strongyloides* spp. aiming at providing more information on the presence of these nematodes in Bornean orangutans under natural and husbandry conditions. The chosen genetic locus contains both slow and fast evolving regions (Baldwin, 1992; Sato *et al*. 2007) providing useful genetic markers for the identification and characterization of *Strongyloides* (Ramachandran *et al*. 1997; Fisher and Viney, 1998; Dorris *et al*. 2002; Sato *et al*. 2007; Hasegawa *et al*. 2009).

To our knowledge, this study provides the first molecular data on infections with *S. f. fuelleborni* and *S. stercoralis* in Bornean orangutans. One isolate identified as *S. f. fuelleborni* originated from a person working at one of the field sites, indicating that wild Bornean orangutans serve as carriers for *S. f. fuelleborni* and that this parasite has a zoonotic potential. In fact, infections with *S. f. fuelleborni* in humans in Borneo might be more common than expected. Further, since the majority of captive

individuals in this study had been living at the centres for 3 weeks up to several years, *S. f. fuelleborni* could have established stable populations within these centres and possibly is transferred back and forth between the animal host and the human caretakers. Indeed, a recent study on intestinal parasites in Bornean orangutans revealed *Strongyloides*-infected human caretakers at 1 of the 2 re-introduction centres also investigated in this study (Labes *et al*. 2010).

Analyses of the ITS1 locus of the *S. f. fuelleborni* isolates revealed huge variability. There was no obvious pattern detectable, neither with respect to geography nor to the status of the host animal of being either captive or semi-captive.

Surprisingly, the sequence of an isolate (no. 240, wild adult male from the central area) identified as *S. f. fuelleborni* at the 18S rRNA locus clustered with sequences of *S. callosciureus* und *S. robustus* and had 99% identity with *S. callosciureus* (GenBank AB272230) at the ITS1 locus. Thus, there seem to be unresolved issues with regard to *Strongyloides* taxonomy (see for example Dorris *et al*. 2002). Indeed, when analysing GenBank entries of the 18S rRNA gene of *Strongyloides* spp., identical sequences are deposited ascribed to *S. f. kellyi* (AJ417029), *S. vituli* (EU885229), *S. papillosus* (AJ417027) and *S. cebus* (AJ417025).

As it was not possible to directly sequence the ITS1 amplicons of isolates no. 5 and 7, sequences were determined of cloned amplicons, revealing considerable variability at this locus, indicating concurrent infections with different genotypes. Thus, analysing single worms instead of pools should be the preferred approach for future such studies.

*Strongyloides stercoralis* was isolated from 1 male orangutan ($\leqslant$8 years of age) which was confiscated from a private household and had arrived at the centre within 1 week prior to sample collection. Thus, the infection had probably been transferred from humans to the ape. Analyses of the ITS1 locus of this isolate revealed, in contrast to the situation in *S. f. fuelleborni*, a very high degree of identity with corresponding sequences of *S. stercoralis* from geographically very distinct locations (East Asia, Caribbean, USA). Hence, the population genetics of these two nematode parasites seems to differ considerably.

The consequences of both *S. f. fuelleborni* and *S. stercoralis* existing in wild and in captive orangutans in Indonesia needs to be further determined with respect to parasite virulence, mortality, risk factors and treatment programmes, as well as the infection risk for humans working closely with this primate.

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